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Molecular Identification of *Streptococcus pyogenes* isolated from tonsilitis in AL –Diwaniyah province

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Abstract

Streptococcus pyogenes(group A streptococcus ,GAS) is a human pathogen that causes a wide spectrum of clinical diseases **.**The pathogenic properties of GAS strains are often linked to the production of virulence factors such as toxins, and M protien.This study included the diagnosis of thirty isolates belonging to the genus *Streptococcuspyogenes*, which were collected from (300) samples from patients with acute and chronic tonsillitis, patients and patients, for both gender and for age groups was (12-45) years of patients returning to the AL –Diwaniyah teaching hospital , children and woman hospital and Afak teaching hospital in Diwaniyah Governorate during the period from 10/1/2022 until 30 /8/2022.

The isolates were diagnosed according to the microbiological, cultural and biochemical characteristics, in addition to the diagnosis of bacitracin allergy, and the diagnosis was confirmed by the Vitekdevice.also used molecular methods to confirm this bacteria depending on *spy1258* gene by PCR technique .this results conferm that *spy*1258gene was definit gene only for Streptococcus pyogenes and can be used as amarker for its detection.The results showed that the percentage of isolation from samples was 22.2% 30 isolates belonging to the genus *Streptococcuspyogenes* .it was investigated for some of the virulence factors molecularly and by using the Polymerase Chain Reaction technique (PCR).The results of the gel electrophoresis showed that all isolates, at a rate of 100%, contained the (*emm*) gene that stimulates the production of M protein.

Introduction

Streptococcus pyogenes group A also called (GAS) They are spherical bacteria, positive for the gram stain, usually containing a capsule composed of hyaluronic acid.It has the ability to ferment lactose, salacin and trihalose and produce acid without releasing gas, decomposing arginine and non-hydrolyzing sodium hiprate or esculin and not having the ability to grow in media containing bile salts at a concentration of 40% or NaCl at a concentration of 6.5%, resistant to optokine, sensitive to bacitracin, and does not grow at a temperature of 10 or 45 °C (Macfaddin, 2000).It has many virulence factors, including M protein, streptokinase, tichoic acid, streptolysine, petidoglycan, hyaluronidase, in addition to the secretion of toxins out of the cell to attack immune cells (Walker *et al*,.2014). Bacteria use virulent factors to enter the body, weaken the immune system's defense mechanisms, and evade the immune system (Ogawa *et al*., 2013).

M- protein

It is a fibrous protein that extends from the surface of the bacterial cell and is encoded by emmgene.Functionally, in the absence of antibodies, the M protein inhibits the complement system, interacts with a large number of host proteins and has a number of inflammatory activities and activities, participates in adhesion to mucous surfaces (Oehmcke*et al*., 2010).

structure of protein M is similar to the protein Staphylococcal A, despite the widespread antigenic variation, all M proteins have an alpha spiral coiled configuration, which forms fibers (about 50-60 nm long), that emerge from the bacterial cell wall (Walker *et al*.,2014). Several studies have shown that M protein molecules are arranged in a hair-like structure under an electron microscope,M proteins are important virulence factors for S. pyogenes discovered by Rebecca Lansfield more than 80 years ago (Le Breton *et al*., 2013; Metzgar and Zampolli, 2011). Protective immunity to S. pyogenes infection is achieved through antibodies directed against the protein (Walker *et al*., 2014).

M protein released by bacteria into the circulatory system can contribute to the systemic activation of the coagulation chain during injury, and soluble platelets can activate the formation of

complexes with neutrophils and monocytes, triggering the activation of these cells, and provoking additional inflammatory responses (La Rhun*et al*., 2019). It consists of a hydrophobic region called (C-terminal), which acts as a membrane anchor through this region, the M protein binds to the bacterial cell wall through it, and is the area rich in amino acids (proline/glycine) (Oehmcke*et al*., 2010). Protein M is also made up of a highly variable region called the N-Terminal that provides antibody protection in this region, which explains the cause of its pathogenicity (La Rhun*et al*., 2019).

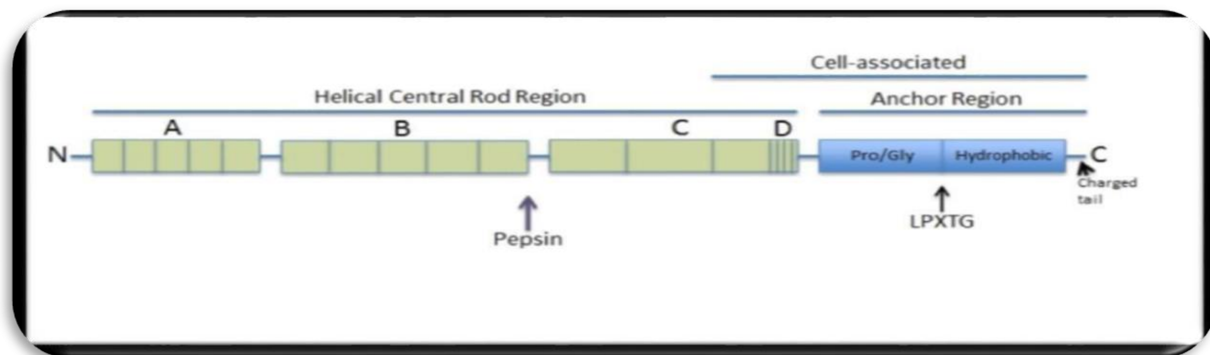
M Protein also gives resistance to phagocytosis by non-immune binding to the FC region of the IgG antibody, moreover, protein M plays an important role in tissue colonization as it has been shown to mediate gas adherence to keratinocytes in the skin by adhering to the CD46 cofactor of the keratinocyte membrane (Metzgar and Zampolli, 2011).

It is extremely polymorphic protein of 41-80 KD in size. In spite of wide range antigenic variation all M proteins have adimeric α – helical coiled – coil configuration , which form fibres (around 50-60 nm in length) emerge outward from the bacterial cell wall (Fischetti , 1989).

They are composed of a hydrophobic region called C- terminal , which acts as a membrane anchor via this region . M - protein is attached to the bacterial cell wall , C- terminal is the conserved region of the molecule and membrane- spanning domain that is rich in amino acid (proline / glycine) (Oehmcke*et al* ., 2010).

The M protein is also composed of hypervariable region called N- Terminal (amino- terminal) . Antibodies of this region confer type-specific protection , and it is the one that accounts for it is pathogenicity (McNamara *et al* ., 2008).

The hyper variable N-terminal region consists of four repeat block or regions that differ in amino acids and size from a strain to another (Oehmcke*et al* ., 2010). The M6 protein structure was studied via (Hollingshead *et al* ., 1986)(Figure 1) and showed that repeat of the As is consisting of 14 amino acid while the B repeats composed of 25 amino acid , C repeats possess 42 amino acid and the D repeats composed of small number of amino acid display some homology from each others (Ferretti*et al* ., 2016).



(Figure 1): M 6 protein sequence shows the 4 repeat blocks of the N- terminal region (Hollingshead *et al* ., 1986).

Materials and Methods

collection of samples

This study was proceeded on three hundred (300)throat samples collected from patients with tonsillitis aged from 12 to 45 years , who refered to AL-Diwaniyah Teaching Hospital , A Hospital for women and children ,Afak General Hospital during the peroid from January 2022 to August 2022 . Patients who presented with fever and phyrngitis were encluded into the study. Throat swab was taken from the pharynx of each patient , and disposable tongue depressor was used to depress the tongue so that the pharynx was apparent , and immediately placed in a trypton soya broth with 5% blood and transferred to the microbiology laboratory , where the broth was incubated at 37℃ for 24 hours .

PCR detection Kits

Table (1): The PCR detection Kits used in this study with their companies and countries of origin:

No.	Kit	Company	Country
1	**Presto™ Mini gDNA Bacteria Kit**	**Geneaid**	**Taiwan**
	Gram+ Buffer		
	GT buffer lysis buffer		
	GB buffer binding buffer		
	Proteinase K		
	W1 buffer		
	Wash buffer		
	Elution buffer		
	GD column		
	Collection tube 2ml		
2	**GoTaq® Green PCR master Mix**	**Promega**	**USA**
	Taq DNA polymerase		
	dNTPs (dATP, dCTP, dGTP, dTTP)		
	Tris-HCl pH 9.0, KCl, & $MgCl_2$		
	Stabilizer and loading dye		

Table (2): Molecular study chemicals and solutions with their company and country of origin:

Chemicals	Company/ Country
Absolute ethanol	CHEM-LAB/ Belgium
DNA Marker ladder 2000-100bp	Bioneer/ Korea
Ethidium bromide	BioBasic/ Canada
TBE buffer	iNtRON / Korea
Agarose	iNtRON / Korea
Free nuclease water	BioLabs/ UK

Table (3): Standard PCR master mix protocol

PCR Master mix	Volume
DNA template 5-100ng	5µL
Forward primer (10pmol)	2µL
Reveres primer (10pmol)	2µL
GoTaq ®Green PCR master	12.5µL
PCR water	3.5µL
Total volume	25 µL

Bacterial DNA extraction
Bacterial genomic DNA was extracted bacterialisolates by using (**Presto™ Mini gDNA Bacteria Kit**) as and done according to company instructions.
Detection of M protein
Primer
The PCR primers that used in This study primers were provided by Scientific Resercher.Co.Ltd in Iraq as following table (4)

Table (4) :primers and product size

Primer	Sequence (5'-3')		Product Size
Spy1258	F	AAAGACCGCCTTAACCACCT	407bp
	R	TGGCAAGGTAAACTTCTAAAGCA	
Emm	F	TATTSGCTTAGAAAATTAA	850-1000bp
	R	GCAAGTTCTTCAGCTTGTTT	

PCR Thermocycler Conditions
PCR thermocycler conditions protocol were done by following table (5)

Table (5): PCR thermocycler conditions protocol

Gene name	Initial denaturation Temp./time	Denaturation Temp./time	Annealing Temp./time	Extension Temp./time	Cycle	Final extension Temp./time	Hold Temp./time
Spy1258	95°C/ 4 min	95°C/ 30 sec	56°C/ 30 sec.	72°C/ 60sec.	32	72°C/ 5min	4°C/forever
emm	95°C/ 4 min	95°C/ 30 sec	59.3°C/ 30 sec.	72°C/ 60sec.	32	72°C/ 5min	4°C/forever

Results and Discussion

Isolation of Streptococcus Pyogenes

In the present study, the results of isolation and diagnosis showed the multiplicity and diversity of bacterial isolates , isolated from the tonsillitis . All the 300 throat swab of patients suffering from tonsillitis (12-45yaers old) were submitted for bacteriological and more of them for molecular study , by using various diagnostic tools to recognize GAS .

Isolates were diagnosed based on the results of bacteriological and biochemical test , using VITEK 2 and by using molecular technique polymerase chain reaction , based on the specific primers amplification was done of highly repeated sequence of DNA of Streptococcus pyogenes .

30 ß – hemolysis isolates were diagnosed which represent 22.2% , 40 isolates of alpha – hemolytic Streptococci 29.6% this finding was in agreement with studies of (Aslan*et al*., 2004) shows that the percentage of Alpha – hemolytic Streptococci is 23.4% isolated from throat swab. 50 isolates of Staphylococcus spp. 37% this result is agreement with AL- Khalifawi (2011) who reported that the percentage of Staphylococcus bacteria which diagnosed is 30.8% while the percentage of other bacteria came slightly lower than

Table (2) No. and percentage of isolated bacteria

Total examined samples	**Streptococcus pyrogens**		**Other bacteria**		**Negative results**	
	No.	**%**	No.	%	No.	%
300	30	10	135	45	135	45

There were 30 isolates were ß-hemolytic , it create 22.2% from total specimen as shown in (figure 2).

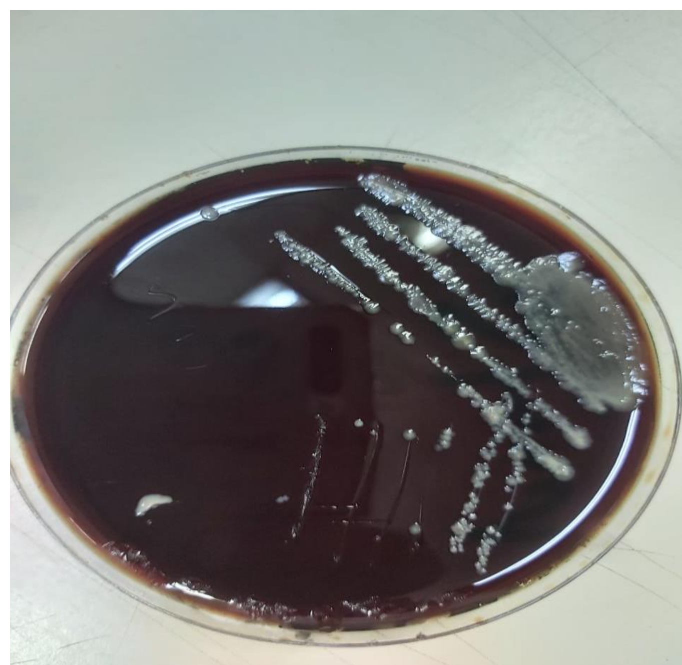


Figure (2): β- hemolytic Streptococcus pyogenes on azide blood agar .

Molecular Diagnosis

Polymerase chain reaction amplification for the detection of the *spy1258* gene:

The results in the current study showed that the occurrence of *Streptococcus pyogenes* 30 from the total 135 isolates . In the present study, the diagnosis of *Streptococcus pyogenes* isolates was confirmed by conventional PCR. The result depicted in (figure 3) reveals the presence of the diagnostic bands of the *spy1258* gene , which has a molecular weight of 407 base pairs in 17 isolates (56.6%) that gave positive result to the VITEK 2 examination. *spy1258* gene was specified gene just for *Streptococcus pyogenes* and used for identification of this bacteria species.this results conferm that *spy*1258gene was definit gene only for *Streptococcus pyogenes* and can be used as amarker for its detection (Dunne *et al*.,2013). These results were in a greement with studies conducted by(Liu *et al*.,2005) this study revealed that *spy1258* gene was particular for GAS only. But not from another species of the genus Streptococcus. This results disagreement with results of (Al-Saadi*et al.*,2015) showed that the SPY1258 gene was present in all *Streptococcus pyogenes* isolates. Also Kumar *et al* (2011) reported that this gene was definite for *streptococcus pyogenes* and could be involved in species to specific reservation or adaptation. This result disagreement with the finding of AL- Shwany and AL- Jebori (2015) who confirmed the presence of *spy1258* gene in all positive isolate for Streptococcus pyogenes and also agreement with those studies performed by Liu *et al* (2005) and Schabauer*et al* (2014) .present study also disagreement with study byKhalaf (2020) reported that this gene was found in all isolates that gave positive result to the VITEK 2 examination . The present study was agreed with the result of the study laid down in Iraq by Degiam*et al*., (2019), which showed that 21 isolates from 24 *Streptococcus Pyogenes* had this gene , and the remain three isolates of *Streptococcus Pyogenes* had lesser sensitivity to detection .

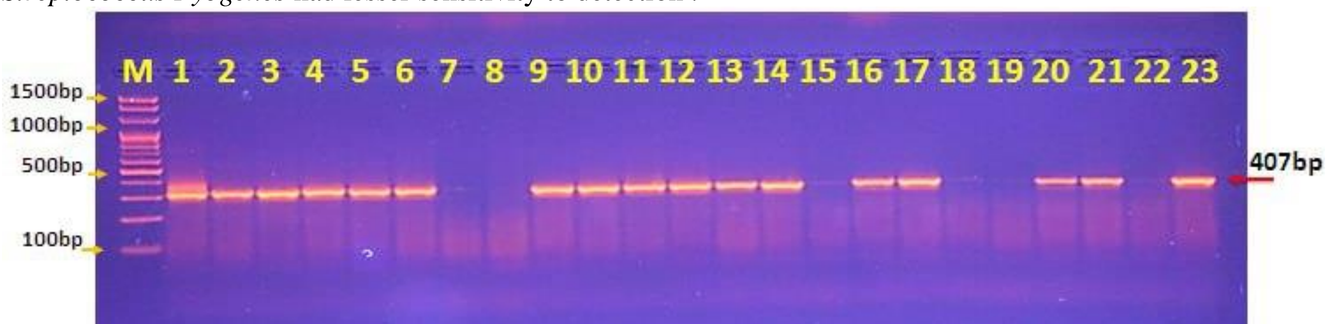


Figure (3): Agarose gel electrophoresis image that showed PCR product analysis for detection of *Spy1258* gene *Streptococcus pyogenes* from tonsillitis patients samples. M (Marker ladder 2000-100bp). Lane (1-23) the positive Spy1258 gene Streptococcus pyogenes isolates at 407bp product.

MolecularDetection of the virulence factor gene M Protein (*emm*)

In order to amplify the emm gene the sets of primers of *emm* F and *emm*R were used. The existence of the *emm* gene was analyzed by using polymerase chain reaction which generated a 850 - 1000 bp bands.The results after removing the isolates from the polymerase chain reaction (PCR) and then placing them in the electrical relay device (Figure 4) gave all the distillations diagnosed were containing the M Protein (*emm*) gene, which numbered 30 bacterial isolates, by 100% and with a size of (850 -1000) bp, the results in this study were not consistent with the study conducted by Khalaf (2020) which obtained 61.5%, and it was almost similar to the study conducted by Koutouzi (2015) in which On the rate of 95.8%, and was also inconsistent with the findings of the researcher Arêas (2014) has obtained 94.5%,also These results were consistent with the study conducted by Hero (2021) in which he obtained 100% .This findings are in contrast to a work done by sambrano*et al*., (2019) who reported a higher ratio for the emm gene 100% from the total 25 isolates ,
and this percentage reached in this study is the highest among the previous percentages .

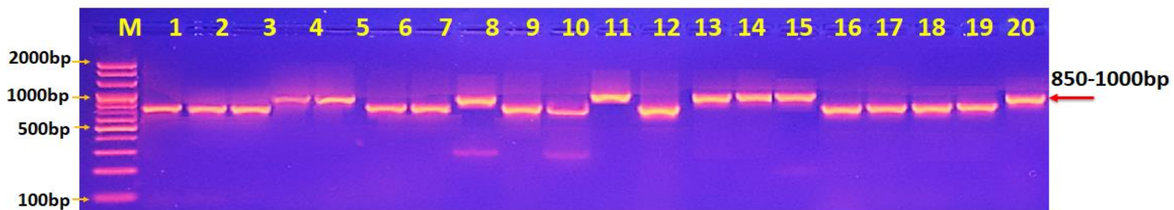


Figure (4): Agarose gel electrophoresis image that showed PCR product analysis for detection of *emm* gene *Streptococcus pyogenes* from tonsillitis patients samples. M (Marker ladder 2000-100bp). Lane (1-20) the positive *emm* gene *Streptococcus pyogenes* isolates at 850 - 1000bp product .

The M protein is of great importance as a vital landmark and can be used in the diagnosis of pathogenic bacterial strains, there are specific regions on the M protein that are common antigens, so their interaction with host proteins (humans) can cause autoimmune diseases such as respiratory, cardiac and skin infections (La Rhun*et al*., 2019). The isolates containing the *emm* gene on the dish are moist or semi-humid form that can be clearly seen under the microscope and the M protein is highly variable and has been used for a long time in epidemiological typological studies of *S. pyogenes* (Le Breton *et al*., 2013). So we can say that the reason may be antibiotic resistance.

Conclusions

It is very important to early identify of Group A streptococcus (GAS) in order to avoid dissemination of the group A streptococcus .*Streptococcus pyogenes* can be identified using the bacitracin sensitive test, however further testing is required for the best results, and the *spy1258* gene should be used as confirmation. With the *spy1258* gene, precise molecular diagnosis is possible.

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